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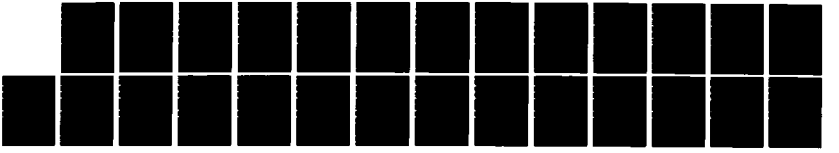

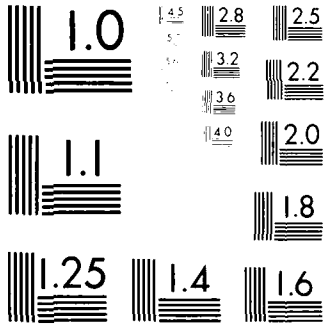

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Absence of Mutagenic Activity of Hycanthone in Serratia marcescens

Gregory B. Knudson[1] and William L. Belser

Department of Biology, University of California, Riverside, California

[1]Present address: U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

Telephone: (301) 663-7341

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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SUMMARY

Hycanthone methanesulfonate, an antischistosomal agent, is an effective frameshift mutagen in Salmonella typhimurium but did not induce auxotrophic mutations in Serratia marcescens HY, even at high concentrations (25,000 µg/ml). The mutagenic action of hycanthone in S. typhimurium is not dependent on a functional excision repair system but is enhanced by the plasmid pKM101, which mediates the inducible error-prone repair system. Hycanthone, like proflavin, intercalates between the stacked bases of DNA. Upon replication, DNA lesions are generated which induce error-prone repair. It is suggested that S. marcescens HY is insensitive to the mutagenic action of hycanthone because it lacks an error-prone repair system.

INTRODUCTION

Schistosomiasis, a chronic illness caused by flukes of the genus Schistosoma, can result in liver and spleen damage or in urinary tract disease. World-wide incidence of schistosomiasis has been estimated at more than 200 million cases (Bueding and Batzinger, 1977), and at least 500 million people are at risk (De Cock, 1984). Lucanthone (Miracil D), and its main metabolic product, hycanthone (Etrenol) are widely used antischistosomal agents (Rosi et al., 1967). Hycanthone methanesulfonate is highly effective against the parasitic worms Schistosoma haematobium and S. mansoni but is ineffective in S. japonicum infections (Berberian et al., 1967; Hillman et al., 1977). The therapeutic dose of hycanthone methanesulfonate is 1.5 to 3.0 mg/kg of body weight, not to exceed 200 mg, given as a single intramuscular injection (Gilles, 1981); serum concentrations of 3 μg/ml are commonly achieved. Batzinger and Bueding (1977) estimated that from 1971 to 1977 over one million people were treated with hycanthone.

Waring (1970) presented evidence, based on changes in the sedimentation coefficient of hycanthone-treated DNA, that the planar aromatic ring structure of hycanthone intercalates between the stacked base pairs of DNA, resulting in uncoiling of the DNA double helix. This was confirmed by Hirschberg and Weinstein (1971), who reported that hycanthone and lucanthone exhibit equivalent activity in increasing the melting temperature and relative viscosity of DNA. Cioli et al. (1985) have recently proposed that hycanthone is activated to form a reactive ester which, upon dissociation, intercalates into and alkylates DNA, forming a covalently bound drug-DNA complex.

Although some biological test systems are ineffective in detecting the mutagenic effects of hycanthone (Arenaz, 1977; Russel and Kelly, 1973; Smith, 1973), overwhelming evidence has accumulated indicating that hycanthone is a potent mutagen (Ong, 1978). Hycanthone was first shown to be a frameshift mutagen in Salmonella typhimurium (Hartman et al., 1971), inducing mutations at a drug concentration as low as 0.1 μg/ml with an estimated 10^5 mutations per 10^8 cells per microgram of hycanthone (Ames et al., 1972; Hartman et al., 1973). Lucanthone produces frameshift mutations in S. typhimurium only after metabolic hydroxylation of its methyl side chain, which converts lucanthone to hycanthone (Hartman et al., 1975; Hernandez et al., 1971). In yeast, hycanthone induces base substitutions (Meadows et al., 1973) and intragenic recombination (Von Borstel and Quah, 1973) in addition to frameshift mutations (Lucchini et al., 1980). Pronounced increases in the frequency of sex-linked recessive lethals in Drosophila melanogaster given a dose of hycanthone of 50 mg/kg body weight have been reported (Knaap and Kramers, 1974; Kramers, 1981; Kramers et al., 1983), while lucanthone was shown to induce chromosome loss in Drosophila male germ cells (U, 1972). Rats given intraperitoneal injections of hycanthone methanesulfonate at 40 mg/kg and killed after 6 hours, showed a significant increase in chromosomal aberrations such as gaps and exchanges in bone marrow cells (Green et al., 1973). Hycanthone methanesulfonate also produced teratogenic effects in mice at doses as low as 30 mg/kg body weight (Moore, 1972), possibly by depressing DNA synthesis at a critical period of organogenesis (Sieber and Adamson, 1975). Hycanthone methanesulfonate was reported to be mutagenic in cultured mouse lymphoma cells at concentrations as low as 5 μg/ml (1×10^{-5} M) for 2 hours (Clive et al., 1972; Clive, 1974), and chromosomal aberrations were produced in cultured human leukocytes after treatment with 5×10^{-8} M hycanthone methanesulfonate for 24 hr (Obe, 1973).

The strong correlation between mutagenesis in bacterial tests and carcinogenesis in animals also applies to hycanthone. An increased incidence of hepatocellular carcinomas was observed in hycanthone-treated mice infected with S. mansoni (Haese et al., 1973; Haese and Bueding, 1976). Rat embryo cell cultures infected with Rauscher leukemia virus are transformed morphologically after treatment with hycanthone at concentrations as low as 0.1 ng/ml (Hetrick and Kos, 1973).

Kohno and Roth (1974) have suggested that proflavin, which has a planar triple ring structure similar to hycanthone, interacts with DNA, which upon replication generates a structure that is recognized by the inducible, recA$^+$ lex$^+$ dependent, error-prone repair pathway (Kohno and Roth, 1974). We have previously reported (G. B. Knudson and W. Belser. 1979. Abstr. Annu. Meet. Am. Soc. Microbiol., H7, p. 120) that Serratia marcescens HY lacks an inducible error-prone repair pathway and that proflavin is ineffective in inducing specific locus mutations in S. marcescens HY. This report extends this study to the mutagenic effects of hycanthone on S. marcescens HY. Salmonella typhimurium his$^-$ tester strains, which lack excision repair, were used as positive controls in testing the mutagenicity of the hycanthone and to assess the effects of the plasmid pKM101 on the mutation frequency.

MATERIALS AND METHODS

Bacterial strains. S. marcescens HY is a wild type strain which has been previously described (Hutchinson and Belser, 1969). All GK-Trp$^-$ mutants were derived from S. marcescens HY by treating log phase cells suspended in tris-maleic buffer (pH 5.9) with N-methyl-N'-nitro-N-nitrosoguanidine at 1 mg/ml for 30 min (Adelberg et al., 1965). S. marcescens GK-16 has a base substitution mutation in the Trp B gene which codes for the beta subunit of tryptophan synthetase. S. marcescens GK-12 has a base substitution mutation in the Trp E gene which codes for anthranilate synthetase. S. marcescens HYC is a strain which was isolated in our lab by curing strain HY of its resident lysogenic bacteriophage with mitomycin C induction followed by growth at high temperatures, as previously described (Kaplan and Brendel, 1969; Steiger, 1973).

Salmonella typhimurium histidine-requiring mutants were kindly supplied by Bruce Ames, Department of Biochemistry, University of California, Berkeley. Strains TA100 (which contains the plasmid pKM101 to increase mutability) and TA1535 were used to test for base-pair substitution mutations. Strains TA100, TA98 (which also contains pKM101), TA1537, and TA1538 were used to detect frameshift mutations. Each strain also lacks the normal lipopolysaccharide cell surface barrier (rfa$^-$) and is excision-repair deficient (uvrB$^-$), which greatly increases their sensitivity to mutagens (Alper and Ames, 1975; Ames et al., 1973, 1975; McCann et al., 1975). All tester strains were routinely confirmed for the uvrB deletion by checking sensitivity to ultraviolet radiation, for the deep rough mutation by checking sensitivity to crystal violet, and for the presence of pKM101 in TA98 and TA100 by ampicillin resistance.

Media and reagents. LB medium is a maximally supplemented broth containing yeast extract and Bacto tryptone (Schleif and Wensink, 1981). SM is a minimal synthetic medium, similar to that used in previous studies (Belser and Bunting, 1956), composed of dibasic potassium phosphate, 8.0 gm; dibasic ammonium citrate, 5.0 gm; magnesium sulfate heptahydrate, 0.5 gm; ferric chloride stock, 1.0 ml (stock consisting of 1 ml molten ferric chloride in 49 ml water); glycerol, 20 ml; and glass-distilled water to make 1.0 liter. SMA medium consists of SM supplemented with 1% vitamin-free acid-hydrolyzed casein. SME medium consists of SM medium supplemented with 2.5 gm peptone, 2.5 gm casitone, and 2.5 gm yeast extract per liter of SM medium. SMME medium consists of SM medium supplemented with 0.02% each of peptone, casitone, and yeast extract. Vogel-Bonner minimal salts medium (Vogel and Bonner, 1956) was supplemented with 1 μg/ml biotin and glucose (autoclaved separately) to give a final concentration of 5 gm per liter and solidified with 1.5% agar (Difco). Top agar consists of 0.6% Difco agar and 0.5% NaCl in glass-distilled water. Before use, 10 ml of filter-sterilized 0.5 mM L-histidine hydrochloride and 0.5 mM biotin is added to 100 ml of melted top agar at 50°C. Adding this supplement allows histidine-requiring bacteria to undergo several rounds of division which, in many cases, is necessary for mutagenesis.

Hycanthone methanesulfonate was a generous gift from F. C. Nachod, Sterling-Winthrop Research Institute, Rensselaer, New York. Fresh filter-sterilized stock solutions of hycanthone methanesulfonate were prepared in saline for each trial.

Preparation of rat-liver S9. A microsome fraction (S9) was prepared from livers of male Sprague-Dawley rats essentially as described by Venitt et al. (1984). Rats were injected i.p. with a polychlorinated biphenyl mixture,

Aroclor 1254 (500 mg/kg), 5 days prior to removing their livers, to induce drug-metabolising enzymes (Alvares et al., 1973). S9 mix (Maron and Ames, 1983) consists of 4% S9 in 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4. S9 mix was made fresh for each mutagenicity assay. Each tester strain was tested with standard diagnostic mutagens (N-methyl-N'-nitro-N-nitrosoguanidine, sodium azide, benzo (a) pyrene, and the acridine half-mustard ICR-191) with and without metabolic activation, as previously described (Pitts et al., 1978).

RESULTS

Spot test for reverse mutation in Serratia marcescens. SM agar plates, supplemented with 0.5 μg/ml tryptophan, were inoculated with 1 x 10^8 S. marcescens trp$^-$ auxotrophs (GK-12 and GK-16). A crystal of nitrosoguanidine was placed in the center of one set of plates and 0.2 mg hycanthone was spotted in the center of a second set of plates. After incubating at 29°C for 3 days, nitrosoguanidine had induced reverse mutations in both GK-12 trp$^-$ and GK-16 trp$^-$, resulting in a ring of red trp$^+$ revertant colonies surrounding a clear zone of inhibition. In contrast, hycanthone failed to revert either trp$^-$ strain of S. marcescens. Furthermore, the hycanthone failed to produce a clear zone of inhibition in the faint lawn of cells that grew on the minimal medium supplemented with a limiting amount of tryptophan.

Endpoint titration. The survival of S. marcescens exposed to increasing concentrations of hycanthone for various times is shown in Fig. 1. Four tubes of SME broth containing hycanthone concentrations of 0, 250, 2500, and 25,000 μg/ml, respectively, were inoculated with 3 x 10^6 log phase cells of S. marcescens HY. After 0, 12, 24, 48, and 96 hours of incubation, serial dilutions were made in saline from each of the four tubes, and 0.1 ml of the appropriate dilutions were plated on SM + 1 μg/ml Trp plates. Cell counts were taken after 4 days of incubation at 29°C. As shown in Fig. 1, there was an increase in the lag phase for cells grown in high concentration of hycanthone. However, there was little toxic effect as reflected in the terminal cell concentration. After incubating 96 hours in SME medium containing 25,000 μg/ml hycanthone, the S. marcescens cell count had reached 1 x 10^{10} cells/ml. The cell count for the control culture, in the absence of

hycanthone, was 4.8×10^{10}. A total of 500 small, putative trp$^-$ colonies were replicate-transferred from these plates to SM minimal plates and to plates of SM + 50 µg/ml Trp. All of these colonies grew on SM plates in the absence of tryptophan, indicating that they were not trp$^-$ auxotrophs.

Ethylenediaminetetraacetate (EDTA) was used to test the possibility that hycanthone was not transported into Serratia due to a permeability barrier (Leive, 1965; Leive and Kollin, 1967). Two strains of S. marcescens (HY and HYC) at log phase in SME were centrifuged and resuspended in tris buffer (pH 8.0, 0.1 M) at 1×10^5 cells/ml. The cell suspension was divided in half; one half was treated with EDTA for 3 min at a final concentration of 0.01 M while the other half served as a control. Hycanthone was added to the EDTA-treated and control cells to give final concentrations of: 0, 50, 500, and 2500 µg/ml. After 24 and 48 hours of incubation at 29°C, the cells were diluted in 0.85% saline, plated on SME, and the plates incubated at 29°C for five days. There were no statistically significant differences in colony counts between the control and EDTA-treated cultures at any of the hycanthone concentrations tested.

Test for forward mutations. Tubes containing SME plus 10 and 500 µg/ml hycanthone, respectively, were inoculated with log phase S. marcescens HYC cells and aerated for 4 hours at 29°C. The cells were centrifuged, resuspended in 0.85% saline, and the appropriate dilutions were plated on SMME agar plates. After incubating at 29°C for five days, the plates were screened for auxotrophic mutants by replicate-transferring all of the very small colonies from the limiting plates (SMME agar plates) to SM, SM + 50 µg/ml Trp, and SME plates. Out of a total of 1000 small colonies tested, none proved to be an auxotrophic mutant.

Ampicillin counterselection. S. marcescens HY was grown in SME containing 25,000 µg/ml nycanthone for 130 hours, centrifuged, washed three times with 0.85% saline, resuspended in SME to Klett 20, and allowed to grow to Klett 40. Growth was monitored turbidimetrically with a Klett-Summerson colorimeter with a red filter (transmission 640 to 700 nm). The cells were centrifuged and suspended in SMA to Klett 10 and grown with aeration for 2.5 hours (to Klett 57) to exhaust their endogenous tryptophan pools. To enrich for trp^- auxotrophs, ampicillin was added to a final concentration of 100 µg/ml, and the cells were aerated for an additional 90 min. The cells were centrifuged and resuspended in distilled water to lyse the fragile trp^+ cells. The cells were diluted in saline and plated on SM + 1 µg/ml Trp plates. After incubating these plates for 5 days at 29°C, 500 small colonies were replicate-transferred to SM and SM + 50 µg/ml Trp plates. All colonies tested grew on both the SM and SM + Trp plates, indicating that none were trp^- mutants.

Test for reversion to histidine independence in Salmonella typhimurium. S. typhimurium tester strains were spot-tested with hycanthone. Log phase cells grown at 37°C in LB-broth on an orbital shaker were adjusted to 1×10^9 cells/ml to reduce experiment-to-experiment variability (Belser et al., 1981). One-tenth ml of cells was added to 2.0 ml molten top agar in a 45°C water batn. Contents were quickly mixed and poured onto a minimal Vogel-Bonner plate which was tilted to evenly distribute the top agar. Crystals of hycanthone were applied directly onto the surface of the plate which was then incubated at 37°C for 2 days. Hycanthone effectively induced reverse mutations in the frameshift tester strains TA1537, TA1538, TA98, and TA100, but not with strain TA1535, which detects base substitutions. On each plate, a ring of his^+ revertant colonies surrounded a zone of inhibition around the

point where the crystals of hycanthone had been applied.

Quantitative tests for hycanthone mutagenesis were carried out by adding hycanthone, at various concentrations, to top agar and the bacterial tester strain, with and without added S9 mix, before pouring on the Vogel-Bonner plates (Maron and Ames, 1983). In each experiment, all strains were plated for spontaneous mutation frequency, and this value was subtracted from the plate counts for each strain. A New Brunswick Biotran II colony counter was used for all plate counts. Hycanthone appeared to be a more potent mutagen in the presence of pKM101. Strain TA98 was derived from strain TA1538 by the addition of the plasmid pKM101. This plasmid enhances error-prone DNA repair. The mutagenic activity of hycanthone at 125 μg/plate was 1.5 times higher with TA98 than with TA1538 (Table 1). By examining the background lawn resulting from the trace of histidine in the top agar, TA1537 and TA1538 appeared to be more sensitive to the toxic effects of hycanthone than TA98. This difference in sensitivity to hycanthone was also seen by comparing the decrease in the number of revertants of TA1537, TA1538, and TA98 at high concentrations of hycanthone (Table 1). There was a linear dose-response for TA1537, TA1538, and TA98 over a range of hycanthone concentrations from 0 to 125 μg/plates (Fig. 2). In agreement with observations of Batzinger and Bueding (1977), rat liver microsomes (S9) did not alter the mutagenic activity of hycanthone.

DISCUSSION

The absence of an inducible error-prone repair pathway in S. marcescens HY was previously demonstrated by showing that it can not support W-reactivation of UV-irradiated phage (Knudson, 1977). Conjugal transfer of plasmid pKM101 into S. marcescens HY mediates enhanced host survival following UV irradiation and supports W-reactivation (Knudson, 1983). This paper reports that hycanthone was not effective in inducing either forward or reverse mutation in derepressed genes of the tryptophan operon of S. marcescens HY. When ampicillin counterselection was used to enrich for trp$^-$ auxotrophs, following incubation with hycanthone, no mutants were detected. These results are consistent with those of Clarke and Shankel (1974), who reported that hycanthone inhibits the excision repair system of E. coli, but does not inhibit, or only weakly inhibits, the action of the error-prone, and hence mutagenic, post-replication repair system.

When EDTA was used to increase the cells permeability to hycanthone, we found that EDTA-treated and untreated cells responded equally to hycanthone, suggesting that penetration of the drug was not a factor in the lack of mutagenesis in S. marcescens HY. At high concentrations (25,000 μg/ml), hycanthone extended the lag period and prolonged the doubling time, but did not affect the final cell concentration at stationary phase.

In confirmation of earlier studies (Hartman et al., 1971, 1973; Cook and Goldman, 1975), we showed that hycanthone is a potent frameshift mutagen in S. typhimurium strains TA98, TA1537, and TA1538. Each of these strains is deficient in DNA excision repair due to a deletion of the uvrB locus. The plasmid pKM101, which enhances inducible error-prone DNA repair, increased

mutagenesis and bacterial survival after hycanthone treatment. At 125 μg/plate, the mutagenic activity of hycanthone was 1.5 time higher in TA98, which contains the plasmid pKM101, than in its parent strain TA1538, which lacks pKM101.

Our data suggest that the lack of sensitivity of S. marcescens HY to hycanthone results from the absence of an inducible error-prone repair system and that a functional excision repair system is not essential for the mutagenic action of hycanthone.

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Table 1. Mutagenic activities of hycanthone on S. typhimurium TA1537, TA1538, and TA98.

	Average Number of Revertants in excess of controls[a]		
Amount	TA1537	TA1538	TA98
μg/plate			
0	0	0	0
3.9	93	86	48
7.8	246	173	78
15.6	387	278	174
31.3	670	411	355
62.5	1409	731	1300
125	2517	1573	2383
250	1426	795	3500
500	0	858	3569

[a]Revertants were computed per 10^8 cells plated. Average titers were 4.6×10^8 cells/ml for TA1537, 6.25×10^8 cells/ml for TA1538, and 5.8×10^8 cells/ml for TA98. Spontaneous reversion values for each strain were subtracted.

Figure 1. Growth inhibitory activity of hycanthone. Survival of Serratia marcescens HY was determined after exposure to hycanthone for various times at: 0 µg/ml (○); 250 µg/ml (●); 2500 µg/ml (△); 25,000 µg/ml (▲). Each point was the average of at least three plates.

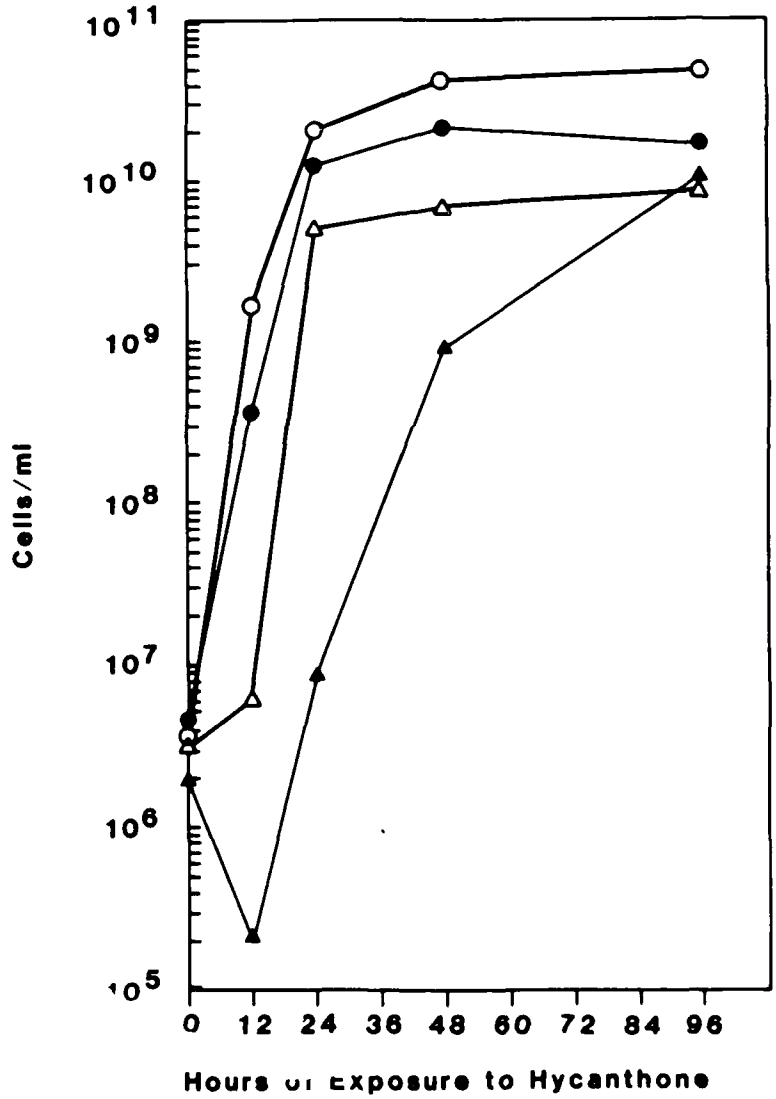
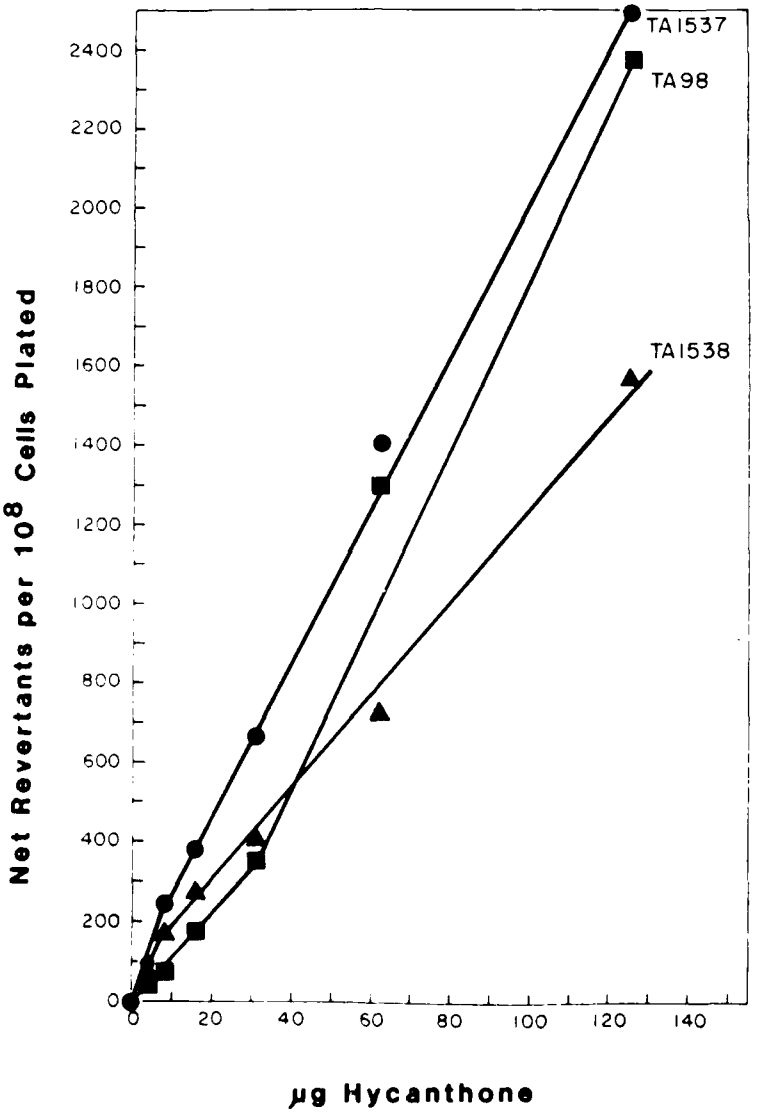


Figure 2. Dose-response curves. Mutagenic activity of hycanthone in the Ames assay is shown with strains TA98 (■), TA1537 (●), and TA1538 (▲). Each point is the mean of at least triplicate plate counts and represents the number of mutants per 10^8 cells/plated, corrected for background mutant frequency.



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